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Isolation and Identification of Lactobacilli from *Garris*, a Sudanese Fermented Camel's Milk Product

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Abstract: In the present study, microbiological quality of *Garris*, a traditional fermented camel's milk product obtained from two production sites in western and central Sudan was investigated. The microbiological analyses showed that Butana *Garris* was found to contain relatively high counts of lactobacilli (8.22±0.28 $\log_{10}$ cfu mL^{-1}) when compared with the counts found in Kordufan *Garris* (7.85±0.45 $\log_{10}$ cfu mL^{-1}). However, Kordufan *Garris* contained relatively higher counts of yeasts (8.42±0.55 $\log_{10}$ cfu mL^{-1}) when compared with that found in Butana *Garris* which contained 7.65±0.32 $\log_{10}$ cfu mL^{-1}). The coliforms averaged 3.2±0.21 and 3.5±0.14 $\log_{10}$ cfu mL^{-1}) in Kordufan and Butana *Garris*, respectively. Twenty strains of lactic acid bacteria were isolated from the fermented milk products and identified based on Polymerase Chain Reaction (PCR) method as belonging to the genus *lactobacillus*. Rapid and reliable two-step multiplex PCR assays were used to identify *Garris* lactobacilli at species level. A multiplex PCR was used for grouping lactobacilli with a mixture of group-specific primers followed by four multiplex PCR assays with four sorts of species-specific primer mixtures for identification at the species level. Primers used were designed from nucleotide sequences of the 16S-23S rRNA intergenic spacer region and its flaking 23S rRNA gene of members of the genus Lactobacillus registered at the GeneBank and DNA Data Bank of Japan. Five lactobacilli isolates from Kordufan *Garris* were identified as *Lactobacillus plantarum*, three isolates as *Lactobacillus paracasei* and two not determined. As for the Butana LAB isolates, five isolates were identified as *Lactobacillus paracasei* subsp *paracasei*, two as *Lactobacillus plantarum* and three were not determined.

Key words: *Garris*, viable microbial counts, lactobacilli, DNA, PCR amplification

INTRODUCTION

Traditional fermented dairy products have been existence in Sudan from a long period of time. These products are widely popular and consumed by a larger section of human population.

Identification of lactic acid bacteria (LAB) lactic acid bacteria mostly depends on traditional phenotypic analyses, although molecular biology-based methods have become available (Hertel *et al*., 1993; Pot *et al*., 1993). Hence, until now modern identification techniques have not been used to a large degree for identification to the species level of lactic acid bacteria from East and Middle African foods.

Garris is a special kind of fermented milk prepared from camel's milk in Sudan. The product is prepared and consumed primarily, by camel's boys, roaming pasturelands. The camel boys taking care of these camels prepare *Garris* on which they live for months as a sole source of nourishment (Dirar, 1993). *Garris* differs from other kinds of Sudanese fermented milks in that it has substantial amounts of ethanol. The product is thus a member of the acid alcoholic fermented milks, which include kefir, koumiss and bukhsa of central Asia (Kosikowski, 1982).

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A semi-continuous fed-batch fermentation process that involves lactobacilli and yeasts, makes *Garris*. Whenever, part of *Garris* is withdrawn for consumption, an equal volume of fresh camel's milk is added. The milk is fermented in two large leather bags covered or imbedded in green grass damped with water. The process of separation of *Garris* and replacement of milk continues for months (Dirar, 1993).

The nutritive value of *Garris* is that of fresh camel's milk as modified by fermentation. Fresh camel milk from the Sudan contains 3.3- 4.7% protein, 2.8-3.6% fat, 4.0- 5.2% lactose, 0.7% ash, 9.2-15.4% solids and to has a pH of 6.0-6.5 (Sulieman *et al*., 2005). In India, it is used to cure dropsy, jaundice, tuberculosis and anaemia. In Russia, camel's milk koumiss has been used to treat tuberculosis and other lung ailments. In Sudan, fermented camel milk is used to cure leishmaniasis and protozoal disease of belly beside many other uses. From the documented literature, it appears that few work has been attempted on assessing the microbiological quality of the Sudanese traditionally fermented milk products. Besides, no study has been initiated identify the fermenting lactobacilli of *Garris*. In this background, the present work aims at isolation and identification of the lactobacilli dominating *Garris* microflora, which are responsible of its fermentation and bringing about its desirable traits.

MATERIALS AND METHODS

Materials

Twenty samples of the Sudanese traditionally fermented camel's milk product, *Garris* were collected from local households in two different sources Kordofan (western Sudan) and Butana (Eastern Sudan) during November 2004. The age of *Garris* samples ranged from 1 to 4 days. The samples were transported in a cooling box to the Department of Food Science and Technology, University of K hartoum, Khartoum, Sudan where the microbiological analyses were performed. Other samples were kept at 4°C, transported by air to the Department of Microbiology, University of Kobe, Japan and kept at low temperature (using a cooling box) temperature pending the isolation and identification analyses.

Viable Microbial Counts

Lactobacilli counts were determined on MRS agar (Merck, Darmstadt, Germany) with glucose a source of energy. Appropriate dilutions were plated on MRS agar (Difco) and incubated anaerobically at 30°C for 48 h using anaerobic jars and gas generating kits (gas pack). Yeasts and moulds were enumerated by surface plating on Potato Dextrose Agar (PDA) (Oxoid) and incubated aerobically at 25 C for 3 days. Coliforms were enumerated by pour plating on MacConkey agar (Oxoid). Characteristic colonies appearing on the respective selective agar media were counted, multiplied by the dilution factor and expressed as colony forming units per milliliter (cfu mL^{-1}).

Isolation of Lactobacilli and Preliminary Tests of the Isolates

MRS was used for isolation of twenty strains of lactobacilli from *Garris* samples, 10 from Kordofan *Garris* and 10 from Botana *Garris*. The isolated strains were purified by random selection of colonies from the MRS agar plates and transformed to tubes containing MRS broth (Oxoid) and incubated again for 24 h at 37 °C. Then, broth cultures were streaked onto MRS agar. The procedure was repeated until purity of the culture was established based on colony appearance.

Preliminary tests were employed on the isolates to ensure that they belong to LAB. These tests included microscopic examination, Gram staining and catalase reaction according to the methods described by Harrigan and McCane (1976). Growth at 15 and 45°C was tested in MRS broth incubated in a Memmert incubator (854 Schwabch, West Germany) and a Salvis water-bath, respectively.

Identification of Lactobacilli at the Genus Level

Identification of Lactobacilli at the genus level was accomplished by a PCR-based method as described by Dubernet *et al* (2002). The reference strains are shown in Table 1

DNA Isolation

For subsequent genotypic analyses, completely genomic DNA from each isolate was prepared following the method described by Marmur (1961). The purity and the amount of DNA in each preparation was estimated colourimetrically and stored at 4°C until use.

Identification of Lactobacilli at the Species Level

The lactobacilli species were identified by PCR assays using group- and species- specific primers derived from 16S-23S rRNA intergenic spacer region and its flaking 23S rRNA as described by Song *et al*. (2000), as follows:

Table 1: Reference strains used for identification of *lactobacilli* at genus level

Species	Strain designation[a]	Origin	Phylogenetic group[b]	Fermentative group[c]	Optimal temperature[d]
L. acidophilus	CNRZ204^T	Human	a	1	37-45°C
L. amylylovorus	CIP102989^T	Cattle	a	1	37-45°C
L. brevis	CIP102806^T	Humanfaeces	b	111	30-37°C
L. buchneri	CIP103023^T	Tomatopuree	b	111	30-37°C
L. caseisubsp. casei	CNRZ313^T	Cheese	b	11	30-37°C
L. cornyformiss ubsp.torquens	CIP103134^T	Aircontaminant (dairybarn)	b	11	30-37°C
L. delbrueckiisubsp.bulgaricus	CIP101027^T	Bulgarian yoghurt	a	1	37-45°C
L. delbrueckiisubsp.delbruckii	CNRZ225^T	Sour grainmash	a	1	37-45°C
L. delbrueckiisubsp.lactis	CNRZ207^T	Cheese (Emmental swiss)	a	1	37-45°C
L. farciminis	CIP103136	Sausage	b	1	30°C
L. fermentum	CNRZ209^T	Fermented beets	b	111	37-45°C
L. fructosus	CIP102985^T	Flowers	c	111	30°C
L. gallinarum	CIP1033611^T	Normal chiken crop	a	1	37°C
L. graminis	CIP105164^T	Grass silage	a	11	3035°C
L. helveticus	CNRZ223^T	Cheese (Emmental swiss)	a	1	37°C
L. johnsonii	CIP103653	ND	a	1	37°C
L. paracaseisubsp.paracasei	CNRZ763	ND	b	11	37-40°C
L. pentosus	CIP103156^T	ND	b	11	30-37°C
L. plantarum	CNRZ211^T	Vinegar cabbage	b	11	30-37°C
L. reuteri	CIP101887^T	Human faeces	b	111	37°C
L. rhamnosus	CIPA157^T	ND	b	11	37°C
L. ruminis	CIP103153^T	Bovine rumen	b	1	30°C
L. suebicus	CIP103411^T	Applemash	b	111	37°C
L. zeae	CIP103253	Cornsteep liquor	ND	11	37°C
E. coli	CIP54.127	ND			30°C
L. lactissubsp. cremoris	CNRZ105^T	ND			30°C
L. lactissubsp. lactis	CNRZ142^T	ND			30°C
L. mesenteroidessubsp. cremoris	CNRZ361^T	lactic starter			25°C
S. thermophilus	CNRZ1358^T	Pasteurized milk			37°C
C. pissicola	CIP103158^T	Kidney			30°C
P. pentosaceus	CIP102260^T	Dried american beer yeast			30°C
B. bifidum	CIP56.7	Child faeces			37°C
W. confusa	DSMZ20196	ND			30°C
Enterococcushirae	CIP58.55	ND			30°C
S. aureus	CIP53.154	ND			37°C
L. monocytogenes	CIP105457	Ovine (brain)		37°	

CND, not determined, CIP[a], Collection de I'institute Pasteur, Paris, France; CNRZ, Centre National de Recherches Zootechniques, Jouy-en-josas, France; DSMZ, Deutsche Sammlung von Mikroorganismen und Zelkultren, Braunschweig, Germany. [b]Hylogenetic group: a, *L. delbruekii* group; b, *L. casei* Pediococcus group; Leuconostoc group. [c]Fermentative group: 1, obligatory homofermentative; 11, facultatively heterofermentative; 111, obligatory heterofermentative

Table 2: Reference strains and the nucleotide sequence accession numbers for the 16S 23S rRNA and its flanking 23S rRNA gene of these strains

Reference strain	Strain No.	Accession No.
Lactobacillus acidophilus	JCM 1132	AF182726
Lactobacillus amylovorus	JCM 1126	AF182732
Lactobacillus brevis	JCM 1059	
Lactobacillus casei	JCM 1134	AF182729
Lactobacillus crispatus	JCM 1185	AF182719
Lactobacillus delbruekii sp. *bulgaricus*	JCM 1002	ABO35485
Lactobacillus delbruekii sp. *delbruekii*	JCM 1012	
Lactobacillus delbruekii sp. *lactis*	JCM 1248	AB035484
Lactobacillus fermentum	JCM 1173	AF182720
Lactobacillus fractivorans	JCM 1173	
Lactobacillus fructosus	JCM 1119	
Lactobacillus gasseri	JCM 1025	AF18271
Lactobacillus helveticus	JCM 1120	AF182728
Lactobacillus jensenii	DSM20557	AB035486
Lactobacillus johnsonni	JCM 2012	
Lactobacillus paracasei sp. *paracasei*	JCM 1181	AF182724
Lactobacillus paracasei sp. *tolerans*	JCM 1171	AB035487
Lactobacillus plantarum	JCM 1149	AF182722
Lactobacillus reuteri	JCM 1112	AF182723
Lactobacillus rhamnosus	JCM 1136	AF182730
Lactobacillus salivarius sp. *salicinius*	JCM 1150	AB035488
Lactobacillus salivarius sp. *salivarius*	JCM 1231	AF182725

Bacterial Strains and Culture Conditions

Twenty two strains of *Lactobacillus* species or subspecies and 84 *lactobacilli* isolated from Japanese stool specimens, which were identified to species level by DNA-DNA hybridization method in a previous study by Song *et al.* (1999), were used as reference strains for identification of lactobacilli at species level (Table 2). All strains were cultured on MRS (Becton Dickinson) at 37°C in an aerobic chamber (Hirasawa, Tokyo, Japan) with an atmosphere consisting of 82%N_2 10%CO_2 and 8% H_2.

PCR Amplification of the 16S-23S rRNA ISR and its Flaking 23S rRNA

A primer pair, 16 and 23-10C (Table 3) (Berthier and Ehrlich, 1998; Gutler and Stanisich, 1996), corresponding to positions of 1526-1543 of the 16S rRNA and positions of 456-474 of the 23S rDNA of *Escherichia coli*, was used to amplify the 16S-23S rRNA ISRs and its flanking 23S rRNA genes of 17 reference strains of the genus Lactobacillus (see strains with the accession numbers in Table 2), which represent Lactobacillus commonly found in human intestinal microflora and their closely related species. PCR amplification was performed as previously described by Kato et al 1991). Briefly, one or two colonies of bacterial strains on an agar plate were suspended in 50 μL of Tris-HCl-EDTA-saline (pH 8.0). The bacterial suspension was incubated for 10 min at 95°C and centrifuged at 186000× g for 2 min to obtain the DNA sample as the PCR template. PCR amplification was performed with a perkin-Elmer DNA thermal cycle 480 (Perkin-Elmer Corporation, Norway, CT, USA) programmed for 35 cycles comprising 95°C for 20 s for denaturation and 55°C for 2 min for annealing and extension.

Sequencing of the PCR-Amplified 16S-23S rRNA Isr and its Flanking 23s rRNA

A major PCR product, an amplicon of about 600-700 bp in size, was excised from a 1% agarose gel after electrophoresis and purified using the Pre-A-Gene purification matrix kit (Bio-Rad, Richmond, CA, USA) and was sequenced directly with ABI PRISM Dye Terminator Cycle Sequencing Really Reaction Kit (PE Applied Biosystems, Foster City, CA, USA) and ABI Prism Genetic Analyzer (PE Applied Biosystems). Cycle sequencing was carried out as recommended by the manufacturer. The analysis of alignment and homology and the construction of a phylogenetic tree for the nucleotide

Table 3: Oligonucleotide primers used in the study

Primer	Sequence (5' 3')	Reference
16	GCTGGATCACCTCCTTTC	Berthier and Ehrlich (1998)
23-10C	CCTTTCCCTCACGGTACTG	Gurtler and Stanisich (1996)
Lde-7	ACAGATGGATGGAGAGCAGA	Present study
LU-1'	ATTGTAGAGCGACCGAGAAG	Present study
LU-3'	AAACCGAGAACACCGCGTT	Present study
LU-5'	CTAGCGGGTGCGACTTTGTT	Present study
Lac-2	CCTCTTCGCTCGCCGCTACT	Present study
Laci-1	TGCAAAGTGGTAGCGTAAGC	Present study
Ljen-3	AAGAAGGCACTGAGTACGGA	Present study
Lcri-3	AGGATATGGAGAGCAGGAAT	Present study
Lcri-2	CAACTATCTCTTACACTGCC	Present study
Lgas-3	AGCGACCGAGAAGAGAGAGA	Present study
Lgas-2	TGCTATCGCTTCAAGTGCTT	Present study
Lfer-3	ACTAACTTGACTGATCTACGA	Present study
Lfer-4	TTCACTGCTCAAGTAATCATC	Present study
Lpla-3	ATTCATAGTCTAGTTGGAGGT	Present study
Lpla-2	CCTGAACTGAGAGAATTTGA	Present study
Lreu-1	CAGACAATCTTTGATTGTTTAG	Present study
Lreu-4	GCTTGTTGGTTTGGGCTCTTC	Present study
Lsal-1	AATCGCTAAACTCATAACCT	Present study
Lsal-2	CACTCTCTTTGGCTAATCTT	Present study
Lpar-4	GGCCAGCTATGTATTCACTGA	Present study
Rha11	GCGATGCGAATTTCTATTATT	Ahrne *et al.* (1998)

sequences obtained in this study were carried out by DNASIS software ver. 3.6 (Hitachi Software Engineering, Yokohama, Japan). Primers for PCR were designed by OLIGO software ver. 4.0 (Hitachi Software Engineering).

Identification of Lactobacilli by Two-step Multiplex PCR Assays

Based on nucleotide sequences of the 16S-23S rRNA ISR of lactobacilli which were determined in this study, lactobacilli were first grouped by multiplex PCR designated multiplex PCR-G) and then identified to species level by four multiplex PCR assay (named multiplex PCR I I-1, multiplex PCR II-2, multiplex PCR III and multiplex PCR IV). Primers used for the grouping and species identification and the aim of each multiplex PCR are given in Table 3.

The DNA sample was extracted as described above. Thirty microliters of a reaction solution for PCR amplification was composed of 0.15 U Taq DNA polymerase (Promega Corporation, Madison, WI, USA), 26.6 μL of a reaction buffer (Promega) supplemented with 200 μM each of dCTP, dATP, dGTP and dTTP (Pharmacia Biotech, Uppsala, Sweden), 10 pmol primer mix comprising one portion of each primer and 0.3 μL of template DNA. PCR was carried out for 35 cycles. Each cycle consisted of 95°C for 20 s for denaturation; annealing and extension was performed for 2 min at 55°C for multiplex PCR-G, 68°C for multiplex PCR II-1, 65°C for multiplex PCR II-2, 62°C for multiplex final extension. Amplicons were analyzed by electrophoresis on a 5% polyacrylamide gel followed by ethidium bromide.

RESULTS AND DISCUSSION

The microbial viable counts of *Garris* samples obtained from the two different production sites are presented in Table 4. Butana *Garris* was found to contain relatively high counts of lactobacilli (8.22±0.28 $\log_{10}$ cfu mL^{-1}) when compared the counts found in B utana *Garris* (7.85±0.45 $\log_{10}$ cfu mL^{-1}). On the other hand, Kordufan *Garris* contained relatively higher counts of yeasts (8.42±0.55 $\log_{10}$ cfu mL^{-1})) when compared with that found in Butana *Garris* which contained 7.65±0.32 $\log_{10}$ cfu mL^{-1}). Presence of yeast in such a high counts suggests that these

Table 4: Microbial viable counts log10 cfu mL^{-1}) and pH of raw milk and *Garris*

	Raw milk	Kordufan *Garris*	Raw milk	Butana *Garris*
Lactobacilli count	6.08±0.12	7.85±0.45	6.11±0.14	8.22±0.28
Yeast count	6.56±0.34	8.42±0.55	6.66±0.44	7.65±0.32
Coliform count	6.7±0.33	3.2±0.21	5.8±0.38	3.5±0.14
pH	6.8±0.15	4.52±0.12	6.78±0.45	4.35±0.13

microorganisms play a role in *Garris* fermentation. Yeast was reported by many investigators as being aroma producer in certain dairy products. Coliforms were found in appreciable amounts in most of the samples. The coliforms averaged 3.2±0.21 and 3.5±0.14 $\log_{10}$ cfu mL^{-1}) in Kordufan and Butana *Garris*, respectively. The pH averaged 4.52±0.12 and 4.35±0.13 for Kordufan *Garris* and Butana *Garris*, respectively.

The pH and microbial counts of raw camel's milk used *Garris* fermentation in both production sites were almost similar with exception to coliform counts which were higher in raw milk used for preparation of Kordufan *Garris* (6.7±0.33 $\log_{10}$ cfu mL^{-1}) when compared with that of Butana *Garris* (5.8±0.38 $\log_{10}$ cfu mL^{-1}). The counts of Lactobacilli and yeasts in raw milk were very high which may lead to the quick spoilage of raw milk in these areas and this puts emphasis on the importance of *Garris* fermentation as a method for cow's milk preservation. However, presence of coliforms and yeasts in such high counts might be due to the improper hygienic conditions during milk handling. However, carry over of large numbers of these microorganisms in *Garris* is a critical health problem because those coliforms might contain pathogenic *E. coli*.

Twenty lactobacillus strains were isolated from *Garris* samples obtained from two different production sites namely Kordofan and Botana. All isolates were Gram-positive, catalse-negative, rods producing no gas from glucose. The strains were identified based on the above characteristics in addition to growth behavior at 15 and 45°C as belonging to the lactic acid bacteria (LAB). On the other hand, PCR-based method revealed that these LAB as belonging to the genus *Lactobacillus*.

On the present study, the authors used a system to identify *Lactobacilli* by two-step multiplex PCR for grouping of lactobacilli followed by a multiplex assays for each group to identify *lactobacilli* at the species level. This is because of the limited variation of the nucleotide sequence targeted and the complexity of multiplex PCR such as selection of specific primers that generate a distinctive PCR product for each species under the same PCR condition.

Among *Lactobacilli* grouped by multiplex PCR *Lactobacillus plantarum* and *Lactobacillus paracasei* subsp *paracasei* were successfully identified at the species level by using one of the four second-step multiplex PCR assays (Table 5). Five lactobacilli isolates from Kordufan *Garris* were identified as *Lactobacillus plantarum*, three isolates as *Lactobacillus paracasei* and two not determined (Table 5). As for the Butana LAB isolates, five isolates were identified as *Lactobacillus paracasei* subsp *paracasei*, two as *Lactobacillus plantarum* and three were not determined. The presence of *Lactobacillus plantarum* was also reported for many African dairy products. Of the identified 21 isolates from naturally fermented milk in Zimbabwe, three were identified as *Lactobacillus plantarum*. From 100 isolates from fermented milk in Northern Tanzania, Isono *et al*. (1994) identified four as *Lactobacillus plantarum*. From cultured milk in Cameroon, Jiwona and Millier (1990) identified 47 out of 426 isolates as *Lactobacillus plantarum*. Sulieman *et al*. (2004) isolated few strains of *Lactobacillus plantarum* from the Sudanese sour milk *Robe*. On the other hand, presence of *Lactobacillus paracasei* subsp *paracasei* is not known in African fermented dairy products (Steinkraus, 1996), but it has been isolated from plant materials and fermented foods (Winter *et al*., 1998; Paludan-Muller *et al*., 1999).

Microbial counts of *Garris* samples obtained from different production sites demonstrated the presence of high counts of *lactobacilli*, yeasts and coliforms. The study demonstrates the high potential for isolating new species of LAB from Sudanese foods. Further investigations into taxonomic classification of the new taxa demonstrated in this study are in progress. In addition, there is a need

Table 5: Strains and results of *Lactobacillus* genus-specific or species-species PCR assays. Identification results based on PCR-based assays

No.	Strain	Source	genus (ref.1)	group (ref.2)	Species
1	SL 1-1	Kordofan *Garris*	±	IV*3	*L. plantarum*
2	SL 1-2	Kordofan *Garris*	±	III*2	*L. plantarum*
3	SL 1-3	Kordofan *Garris*	±	III	*L. paracasei*
4	SL 1-4	Kordofan *Garris*	±	III	*L. paracasei*
5	SL 1-5	Kordofan *Garris*	±	-	*L. paracasei*
6	SL 1-6	Kordofan *Garris*	±	IV	ND
7	SL 1-7	Kordofan *Garris*	±	IV	*L. plantarum*
8	SL 1-8	Kordofan *Garris*	±	IV	*L. plantarum*
9	SL 1-9	Kordofan *Garris*	?	?	ND
10	SL 1-10	Kordofan *Garris*	±	-	*L. plantarum*
11	SL 1-1	Botana *Garris*	±	III	ND
12	SL 1-2	Botana *Garris*	±	III	ND
13	SL 1-3	Botana *Garris*	±	III	*L. paracasei*
14	SL 1-4	Botana *Garris*	±	IV	*L. paracasei*
15	SL 1-5	Botana *Garris*	±	III	*L. plantarum*
16	SL 1-6	Botana *Garris*	±	III	*L. paracasei*
17	SL 1-7	Botana *Garris*	±	III	*L. paracasei*
18	SL 1-8	Botana *Garris*	±	III	ND
19	SL 1-9	Botana *Garris*	±	IV	*L. paracasei*
20	SL 1-10	Botana *Garris*	±	-	*L. plantarum*[*1]

ND: not tested, *2 Group III includes *Lactobacillus paracasei* subsp. *paracasei, L. paracasei* subsp.*casei, L. paracasei* subsp. *tolerans* and *L. rhamnosus*. *3 Group IV includes *Lactobacillus plantarum, L. reuteri, L. salivarius* and *L. fermentum*

for investigating the technological characteristics of *Garris* dominant microorganisms to select the most appropriate strains as starter culture for a controlled fermentation process.

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